(51) International Patent Classification 5 : A01N 25/26, 25/28, 43/04, A61K 31/70, 35/14, 49/00, C07K 3/00, 13/00, 15/00, C12N 15/00, C12P 21/02, 21/06, G01N 31/00, 33/48	A1	(11) International Publication Number: WO 94/27435 (43) International Publication Date: 8 December 1994 (08.12.94)

(21) International Application Number: PCT/US94/06105

(22) International Filing Date: 31 May 1994 (31.05.94)

(30) Priority Data:
    08/069,720   1 June 1993 (01.06.93)   US

(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 8717 Grovemont Circle, P.O. Box 6009, SG2-Corp. R & D, Gaithersburg, MD 20877 (US).

(72) Inventors: JESSEE, Joel, A.; 4139 Old National Pike, Mt. Airy, MD 21771 (US). HEARL, William, G.; 5728 Cedar Lane, Columbia, MD 21044 (US).

(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Gold-stein & Fox, Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
   With international search report.

(54) Title: GENETIC IMMUNIZATION WITH CATIONIC LIPIDS

(57) Abstract

    A method for immunization using genetic material is disclosed. Compositions for genetic immunization comprising cationic lipids and polynucleotides are also disclosed. Methods for using genetic immunization to produce polyclonal and monoclonal antibodies are also disclosed. A method for epitope mapping is also disclosed.

Genetic Immunization with Cationic Lipids

Field of the Invention

The present invention is in the field of immunology. In particular, this invention is directed to methods of immunization using compositions comprising cationic lipids and polynucleotide molecules which code for immunogens. This invention is also directed to methods for producing polyclonal and monoclonal antibodies from genetically immunized animals. This invention is further directed to the use of genetic immunization to map protein epitopes.

Background of the Invention

Traditional methods of immunization are achieved by injection of a mixture of antibodies which immunoreact with an invading pathogen (i.e., passive immunization), or by vaccination, which stimulates the immune system to produce pathogen-specific antibodies. Since foreign antibodies are cleared by the recipient, passive immunity confers only temporary protection. Vaccination confers longer-lasting active immunity.

In order to be effective, vaccination must generate humoral and/or cell-mediated immunity which will prevent the development of disease upon subsequent exposure to the corresponding pathogen. The pertinent antigenic determinants must be presented to the immune system in a manner that mimics

- 2 -

a natural infection. Conventional viral vaccines may consist of inactivated virulent strains, or live-attenuated strains (Old *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, Blackwell Scientific Publications, 4th edition, 1989). A general problem with using a vaccine consisting of a virus is that many viruses (such as hepatitis B virus) have not been adapted to grow to high titre in tissue culture and thus, cannot be produced in sufficient quantity (*Id.*). In addition, the use of inactivated viruses present a potential danger of vaccine-related disease resulting from replication-competent virus may remain in the inoculum. Outbreaks of foot-and-mouth disease in Europe have been attributed to this cause (*Id.*). On the other hand, attenuated virus strains have the potential to revert to virulent phenotype upon replication in the vaccinee. This problem has been reported to occur about once or twice in every million people who receive live polio vaccine (*Id.*). Moreover, encephalitis can occur following measles immunization with attenuated virus (Roit, I.M. *Essential Immunology*, Blackwell Scientific Publications, Sixth Ed., 1988). Another disadvantage of using attenuated strains is the difficulty and expense of maintaining appropriate cold storage facilities (*Id.*). A major disadvantage associated with the use of live virus vaccines is that persons with congenital or acquired immunodeficiency risk severe infections. Such persons include children in developing countries who are often immunodeficient because of malnutrition and/or infection with viruses or parasites (*Id.*, Old *et al.*, *supra*).

As a result of recent advances in molecular biology and peptide synthesis, it is possible to produce purified viral proteins or synthetic peptides for use in immunoprophylaxis (Murphy *et al.*, "Immunization Against Viruses," in *Virology*, Fields *et al.*, Eds., Raven Press, New York, pp. 349-370, 1985). Purified antigens may be produced by synthesizing peptides which represent immunologically important domains of surface antigens of the pathogen. The synthetic peptide approach has been successfully used with an antigenic determinant of the foot and mouth disease virus (*Id.*). One problem with this approach is that the poor antigenicity of synthetic peptides has

required the use of Freund's adjuvant to enhance the immune response in experimental animals (*Id.*). Since Freund's adjuvant cannot be used in humans, an effective adjuvant for human use must be developed (*Id.*). In addition, a single antigenic site may not be sufficient to induce resistance since large surface antigens usually contain several distinct immunological domains that elicit a protective humoral and/or cell-mediated response (Braciale *et al.*, *J. Exp. Med.* 153:910-923 (1981); Wiley *et al.*, *Nature* 289:373-378 (1981)). There may also be difficulties in stimulating an immunologic response to epitopes that are formed by noncontiguous parts of the linear protein molecule (Murphy, *et al.*, *supra*). There is evidence that the majority of protein determinants are discontinuous and involve amino acid residues that are far apart in the primary amino acid sequence, but are brought into close juxtaposition by peptide folding (Roit, *supra*).

The alternative approach to preparing proteins for vaccines involves the use of cloned viral DNA inserted into a suitable vector to produce viral protein in prokaryotic or eukaryotic cells (Aldovini *et al.*, *The New Vaccines*, *Technology Review*, pp. 24-31, January 1992). This approach, also, has several limitations. For example, one must devise suitable conditions for the optimal production of the recombinant protein of interest by the recombinant host cells. The protein product must be isolated and purified from the culture system, and obtained in sufficient quantities for use as a vaccine. Finally, it may be necessary to perform post-translational modifications of the purified protein (such as glycosylation and/or cleavage of a fusion protein).

An alternative to producing the recombinant antigen *in vitro* is to introduce nucleic acid sequences coding for the antigen into the cells of the vaccinee. In this way, the antigen is produced *in vivo* by the vaccinee's cells and provokes the immune response. Tang *et al.* (*Nature* 356:152-154 (1992)) have shown that it is possible produce an immune response to human growth hormone protein in mice by propelling gold microprojectiles coated with plasmids containing human growth hormone genomic sequences. The resultant variability in the production of antibody production was hypothesized to arise

- 4 -

from the operation of the microprojectile device, or the coating of the DNA onto the microprojectiles.

More recently, Ulmer *et al.* (*Science* 259:1745-1749 (1993)) injected a plasmid carrying the gene for influenza A nucleoprotein into the quadriceps of mice. The mice produced nucleoprotein antibodies, indicating that the gene was expressed in murine cells. The mice also produced nucleoprotein-specific cytotoxic T lymphocytes which were effective in protecting the mice from a subsequent challenge with a heterologous strain of influenza A virus. Similarly, Wang *et al.* (*Proc. Natl. Acad. Sci. USA* 90:4156-4160 (1993)) observed that the intramuscular injection of a human immunodeficiency virus (HIV) type 1 envelope DNA construct in mice generated antigen-specific cellular and humoral immune responses. In addition, splenic lymphocytes derived from the inoculated mice demonstrated HIV-envelope-specific proliferative responses. Thus, direct inoculation of DNA coding for pathogenic antigens can provide an alternative to the use of viruses, proteins, or peptides.

One problem with using naked DNA for inoculation is the low efficiency of cellular uptake. For example, the protocol of Wang *et al.*, *supra*, requires the injection of 100 μg of the DNA construct biweekly for a total of four inoculations. As described herein, the use of cationic lipids as a carrier for DNA constructs provides a more efficient means of genetic immunization. According to the present invention, genetic immunization can be achieved with as little as 5 μg of a DNA construct, which has been complexed with cationic lipid.

Liposomes have been used as carriers of genetic information in the transfection of tissue culture cells. A fundamental problem of liposome-mediated transfection with liposomes comprising neutral or anionic lipids is that such liposomes do not generally fuse with the target cell surface. Instead, the liposomes are taken up phagocytically, and the polynucleotides are subsequently subjected to the degradative enzymes of the lysosomal compartment (Straubinger *et al.*, *Methods Enzymol.* 101:512-527 (1983);

Mannino *et al.*, *Biotechniques* 6:682-690 (1988)). Another problem with conventional liposome technology is that the aqueous space of typical liposomes may be too small to accommodate large macromolecules such as DNA or RNA. As a result, typical liposomes have a low capturing efficiency (Felgner, "Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)).

Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture 100% of the polynucleotide (Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417 (1987); Felgner *et al.*, *Focus* 11:21-25 (1989)). Moreover, the polycationic complexes are taken up by the anionic surface of tissue culture cells with an efficiency that is about ten to one hundred times greater than negatively charged or neutral liposomes (Felgner, "Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)). In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment (Düzgünes *et al.*, *Biochemistry* 28:9179-9184 (1989); Felgner *et al.*, *Nature* 337:387-388 (1989)).

Various formulations of cationic lipids have been used to transfect cells *in vitro* (WO 91/17424; WO 91/16024; U.S. Patent No. 4,897,355; U.S. Patent No. 4,946,787; U.S. Patent No. 5,049,386; and U.S. Patent No. 5,208,036). Cationic lipids have also been used to introduce foreign polynucleotides into frog and rat cells *in vivo* (Holt *et al.*, *Neuron* 4:203-214 (1990); Hazinski *et al.*, *Am. J. Respr. Cell. Mol. Biol.* 4:206-209 (1991)). Therefore, cationic lipids may be used, generally, as pharmaceutical carriers to provide biologically active substances (for example, *see* WO 91/17424; WO 91/16024; and WO 93/03709). Thus, cationic liposomes can provide an

- 6 -

efficient carrier for the introduction of foreign polynucleotides into host cells for genetic immunization.

Various cationic lipids are well-known in the prior art. One well-known cationic lipid is N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). The structure of DOTMA is:
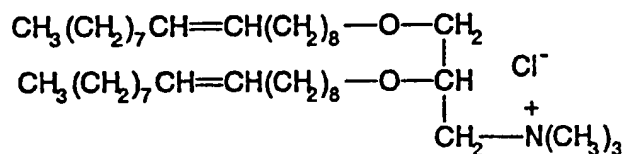
$$CH_3(CH_2)_7CH{=}CH(CH_2)_8{-}O{-}CH_2$$
$$CH_3(CH_2)_7CH{=}CH(CH_2)_8{-}O{-}CH \quad Cl^-$$
$$CH_2{-}\overset{+}{N}(CH_3)_3$$

DOTMA, alone or in a 1:1 combination with dioleoylphosphatidylethanolamine (DOPE) can be formulated into liposomes using standard techniques. Felgner *et al.* (*Proc. Natl. Acad. Sci. U.S.A. 84*:7413-7417 (1987)) have shown that such liposomes provide efficient delivery of nucleic acids to cultured cells. A DOTMA:DOPE (1:1) formulation is sold under the name LIPOFECTIN™ (GIBCO/BRL: Life Technologies, Inc., Gaithersburg, MD). Another commercially available cationic lipid is 1,2-bis(oleoyloxy)-3-3-(trimethylammonia)propane (DOTAP), which differs from DOTMA in that the oleoyl moieties are linked via ester bonds, not ether bonds, to the propylamine. DOTAP is believed to be more readily degraded by target cells.

A related groups of known compounds differ from DOTMA and DOTAP in that one of the methyl groups of the trimethylammonium group is replaced by a hydroxyethyl group. Compounds of this type are similar to the Rosenthal Inhibitor of phospholipase A (Rosenthal *et al.*, *J. Biol. Chem. 235*:2202-2206 (1960), which has stearoyl esters linked to the propylamine core. The dioleoyl analogs of the Rosenthal Inhibitor (RI) are commonly abbreviated as DORI-ether and DORI-ester, depending upon the linkage of the fatty acid moieties to the propylamine core. The hydroxy group can be used

- 7 -

as a site for further functionalization, for example, by esterification to carboxyspermine.

Another class of known compounds has been described by Behr *et al.* (*Proc. Natl. Acad. Sci. USA* *86*:6982-6986 (1989); EPO Publication 0 394 111), in which carboxyspermine has been conjugated to two types of lipids. The structure of 5-carboxylspermylglycine dioctadecylamide (DOGS) is:

$$R-N-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-N-\overset{\overset{\displaystyle O}{\|}}{C}-CH(CH_2)_3NH(CH_2)_3NH_2$$

$$NH(CH_2)_3NH_2$$

$$R = CH_3(CH_2)_{17}$$

The structure of dipalmitoylphosphatidylethanolamine 5-carboxyspermylamide (DDPES) is:

$$R-\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_2$$

$$R-\overset{\overset{\displaystyle }{}}{C}-O-CH \quad \quad \quad R = CH_3(CH_2)_{15}$$

$$\overset{\|}{O} \quad CH_2-O-\overset{\overset{\displaystyle O}{\|}}{P}-O-CH_2-CH_2NH-\overset{\overset{\displaystyle O}{\|}}{C}-CH-CH(CH_2)_3NH(CH_2)_3NH_2$$

$$\overset{|}{O^-} \quad \quad NH(CH_2)_3NH_2$$
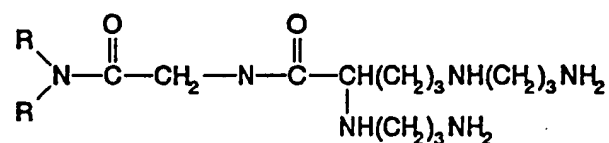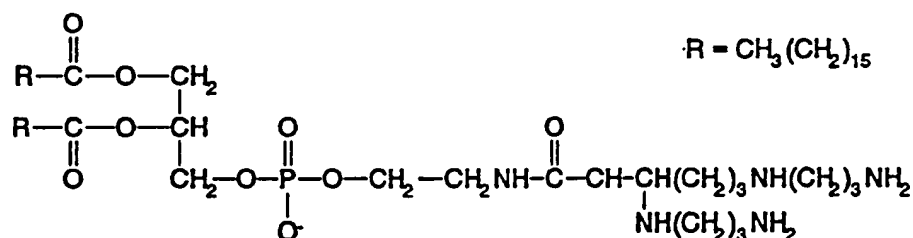
Both DOGS and DPPES have been used to coat plasmids, forming a lipid aggregate complex that provides efficient transfection. The compounds are claimed to be more efficient and less toxic than DOTMA for transfection of certain cell lines. DOGS is available commercially as TRANSFECTAM™ (Promega, Madison, WI).

A cationic cholesterol derivative (DC-Chol) has been synthesized and formulated into liposomes in combination with DOPE (Gao *et al., Biochim.*

Biophys. Res. Comm. 179:280-285 (1991)). The structure of this compound is:

$$\begin{array}{c} CH_3 \\ \diagdown \quad + \\ \diagup N H - (CH_2)_2 NH - \overset{\displaystyle O}{\overset{\|}{C}} - [Cholesterol] \\ CH_3 \end{array}$$
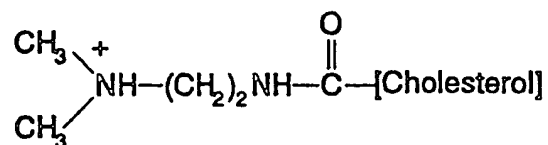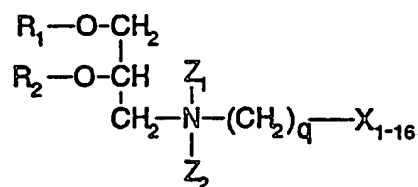
Liposomes formulated with DC-Chol provide more efficient transfection and lower toxicity than DOTMA-containing liposomes for certain cell lines.

Lipopolylysine is formed by conjugating polylysine to DOPE. This compound has been reported to be especially effective for transfection in the presence of serum (Zhou *et al.*, *Biochim. Biophys. Res. Comm. 165*:8-14 (1991)). Thus, lipopolylysine may be an effective carrier for immunization.

In addition, Gebeyhu *et al.* (co-pending U.S. application serial no. 07/937,508; filed August 28, 1992) have developed novel cationic lipids according to the general formula:

$$\begin{array}{l} R_1 - O - CH_2 \\ R_2 - O - CH \quad Z_1 \\ \qquad \quad | \qquad | \\ \qquad \quad CH_2 - N - (CH_2)_q - X_{1-16} \\ \qquad \qquad \quad | \\ \qquad \qquad \quad Z_2 \end{array}$$

wherein R_1 and R_2 separately or together are C_{1-23} alkyl or

or $-\overset{\displaystyle O}{\overset{\|}{C}} - C_{1-23}$ alkyl or alkenyl, q is 1 to 6,

Z_1 and Z_2 separately or together are H or unbranched alkyl C_{1-6}
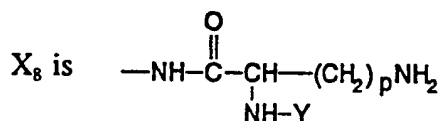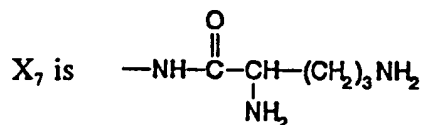
X_1 is $-(CH_2)_n Br$, Cl, F or I   n=0-6 or

X_2 is $-(CH_2)_n NH_2$ n=0-6 or

X_3 is $-NH-(CH_2)_m-NH_2$ m=2-6 or

X_4 is $-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$ or

X_5 is $-NH-(CH_2)_3-NH-(CH_2)_4-NH(CH_2)_3-NH2$

$$X_6 \text{ is} \quad -NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH-(CH_2)_3 NH_2}{|}}{CH}-(CH_2)_3 NH(CH_2)_3-NH_2$$

$$X_7 \text{ is} \quad -NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH_2}{|}}{CH}-(CH_2)_3 NH_2$$

$$X_8 \text{ is} \quad -NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH-Y}{|}}{CH}-(CH_2)_p NH_2$$

where p is 2-5, Y is H or other groups attached by amide or alkyl amino group or

X_9 is a polyamine, e.g., polylysine, polyarginine, polybrene, histone or protamine or

$$X_{10} \text{ is a reporter molecule, e.g.,} \quad -NH-\overset{\overset{\displaystyle O}{\|}}{C}-\text{fluorescein, biotin, folic}$$

acid or PPD, or

X_{11} is a polysaccharide or substituted polysaccharide, or

X_{12} is a protein or

X_{13} is an antibody or

X_{14} is an amine or halide reactive group or

X_{15} is $-(CH_2)_r$-SH where r is 0-6 or

X_{16} is $-(CH_2)_s$-S-S-$(CH_2)_t$-NH_2 where s is 0-6 and t is 2-6.

These compounds are useful either alone, or in combination with other lipid aggregate-forming components (such as DOPE or cholesterol) for formulation into liposomes or other lipid aggregates. Such aggregates are cationic and able to complex with anionic macromolecules such as DNA or RNA.

- 10 -

Summary of the Invention

The present invention is directed to a method for eliciting an immune response in an animal, comprising the steps of: (a) mixing at least one cationic lipid with a polynucleotide, coding for an antigenic determinant, thereby forming a cationic lipid-polynucleotide complex; and (b) administering the lipid-polynucleotide complex to the animal.

The present invention is also directed to a method for generating active immunity against an infectious disease in an animal, comprising the steps of: (a) mixing at least one cationic lipid with a polynucleotide, coding for an antigenic determinant of an organism which is the causative agent of the infectious disease, thereby forming a cationic lipid-polynucleotide complex; and (b) administering the lipid-polynucleotide complex to the animal; whereby active immunity to the infectious disease is generated.

The present invention is also directed to such a genetic immunization method wherein the polynucleotide is an expression vector comprising a DNA sequence coding for an immunogen, wherein the transcription of the DNA sequence is under the control of a promoter.

The present invention is further directed to a genetic immunization method wherein the polynucleotide is an RNA molecule which codes for an immunogen.

The present invention is further directed to a method for producing polyclonal antibodies comprising the use of the genetic immunization method described above, and further comprising the step of isolating the polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies comprising the steps of:

(a)   mixing at least one cationic lipid with a polynucleotide thereby forming a lipid-polynucleotide complex, wherein the polynucleotide comprises a DNA sequence coding for an immunogen;

(b)   administering the lipid-polynucleotide complex to at least one mouse;

(c)   removing B-lymphocytes from the immunized mice;

(d)   fusing the B-lymphocytes from the immunized mice with myeloma cells, thereby producing hybridomas;

(e)   cloning the hybridomas;

(f)   selecting positive clones which produce anti-immunogen antibody;

(g)   culturing the anti-immunogen antibody-producing clones; and

(h)   isolating anti-immunogen antibodies from the cultures.

The present invention is also directed to a method for mapping the epitopes of a protein molecule, comprising the steps of:

(a)   fragmenting DNA molecules coding for the protein in a random manner;

(b)   subcloning the DNA fragments in an expression vector;

(c)   mixing at least one cationic lipid with each expression vector subclone, thereby forming a cationic lipid-expression vector complex with each expression vector subclone;

(d)   administering the cationic lipid-expression vector complexes to mice; and

(d)   determining which of the DNA fragments are capable of generating the production of antibodies in the mice.

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

- 12 -

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

Recombinant Host. In general, a recombinant host may be any prokaryotic or eukaryotic microorganism or cell which contains the desired cloned genes on an expression vector or cloning vector. This term is also meant to include those microorganisms that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism.

Recombinant vector. Any cloning vector or expression vector which contains the desired cloned gene(s).

Host. Any prokaryotic or eukaryotic microorganism or cell that is the recipient of a replicable expression vector or cloning vector. A "host," as the term is used herein, also includes prokaryotic or eukaryotic microorganisms or cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, *see* Maniatis *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

Promoter. A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible

promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Transfection. Transfection refers to the transformation of a host cell with DNA. The recombinant host cell expresses protein which is encoded by the transfected DNA.

Epitope. The part of a non-immunoglobulin antigen to which the variable region of an antibody binds.

Antigenic Determinant. A protein or peptide which contains one or more epitopes.

Immunogen. A protein or peptide which is capable of eliciting an immune response due to the presence of one or more epitopes.

- 14 -

Detailed Description of the Invention

The present invention is directed to a method for eliciting an immune response in an animal by administering a cationic lipid-polynucleotide complex, wherein the polynucleotide codes for an antigenic determinant.

The present invention is also directed to a method for generating active immunity against an infectious disease in an animal by administering a cationic lipid-polynucleotide complex, wherein the polynucleotide codes for an antigenic determinant of an organism which is the causative agent of the infectious disease.

The present invention is also directed to such a genetic immunization method wherein the polynucleotide is an expression vector comprising a DNA sequence coding for an immunogen, wherein the transcription of the DNA sequence is under the control of a promoter.

The present invention is further directed to a genetic immunization method wherein the polynucleotide is an RNA molecule which codes for an immunogen.

The present invention is further directed to a method for producing polyclonal antibodies comprising the use of the genetic immunization method described above, and further comprising the step of isolating the polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies using B-lymphocytes from mice following genetic immunization.

The present invention is also directed to a method for epitope mapping using genetic immunization.

I. Cationic Liposomes

Any of the cationic lipids known in the prior art may be employed in the practice of the claimed invention. *See*, for example, Felgner *et al.* (*Proc. Natl. Acad. Sci. U.S.A. 84*:7413-7417 (1987)); Felgner *et al.* (*Focus 11*:21-25 (1989)); Felgner ("Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)); WO 91/17424; WO 91/16024; U.S. Patent No. 4,897,355; U.S. Patent No. 4,946,787; U.S. Patent No. 5,049,386; U.S. Patent No. 5,208,036; Behr *et al.* (*Proc. Natl. Acad. Sci. USA 86*:6982-6986 (1989); EPO Publication 0 394 111); Gao *et al.* (*Biochim. Biophys. Res. Comm. 179*:280-285 (1991)); Zhou *et al.*, (*Biochim. Biophys. Res. Comm. 165*:8-14 (1991)); and Gebeychu *et al.* (co-owned U.S. application serial no. 07/937,508; filed August 28, 1992), the contents of which are fully incorporated by reference.

Preferred cationic lipids include N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). The structure of DOTMA is:
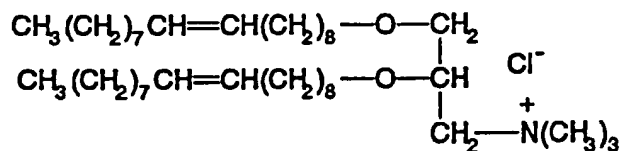
$$CH_3(CH_2)_7CH=CH(CH_2)_8—O—CH_2$$
$$CH_3(CH_2)_7CH=CH(CH_2)_8—O—CH \quad Cl^-$$
$$CH_2—N(CH_3)_3^+$$

DOTMA, alone or in a 1:1 combination with dioleoylphosphatidylethanolamine (DOPE) can be formulated into liposomes using standard techniques. A DOTMA:DOPE (1:1) formulation is sold under the name LIPOFECTIN™ (GIBCO/BRL: Life Technologies, Inc., Gaithersburg, MD).
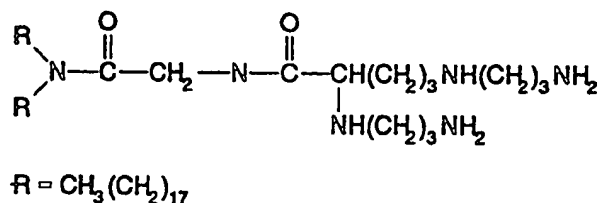
Another preferred commercially available cationic lipid is 1,2-bis(oleoyloxy)-3-3-(trimethylammonia)propane (DOTAP), which differs from

- 16 -

DOTMA in that the oleoyl moieties are linked via ester bonds, not ether bonds, to the propylamine.

A related group of preferred cationic lipids differ from DOTMA and DOTAP in that one of the methyl groups of the trimethylammonium group is replaced by a hydroxyethyl group. Compounds of this type are similar to the Rosenthal Inhibitor of phospholipase A (Rosenthal *et al., supra*), which has stearoyl esters linked to the propylamine core. The dioleoyl analogs of the Rosenthal Inhibitor (RI) are commonly abbreviated as DORI-ether and DORI-ester, depending upon the linkage of the fatty acid moieties to the propylamine core. The hydroxy group can be used as a site for further functionalization, for example, by esterification to carboxyspermine.

In another class of preferred cationic lipids, carboxyspermine has been conjugated to two types of lipids. The structure of 5-carboxylspermylglycine dioctadecylamide (DOGS) is:

$$R\!\!-\!\!N\!\!-\!\!\overset{\overset{\displaystyle O}{\|}}{C}\!\!-\!\!CH_2\!\!-\!\!N\!\!-\!\!\overset{\overset{\displaystyle O}{\|}}{C}\!\!-\!\!CH(CH_2)_3NH(CH_2)_3NH_2$$
$$\underset{\displaystyle NH(CH_2)_3NH_2}{|}$$

$$R = CH_3(CH_2)_{17}$$

The structure of dipalmitoylphosphatidylethanolamine 5-carboxyspermylamide (DDPES) is:

$$R-C(=O)-O-CH_2$$
$$R-C(=O)-O-CH$$
$$CH_2-O-P(=O)(O^-)-O-CH_2-CH_2NH-C(=O)-CH-CH(CH_2)_3NH(CH_2)_3NH_2$$
$$NH(CH_2)_3NH_2$$

$$R = CH_3(CH_2)_{15}$$
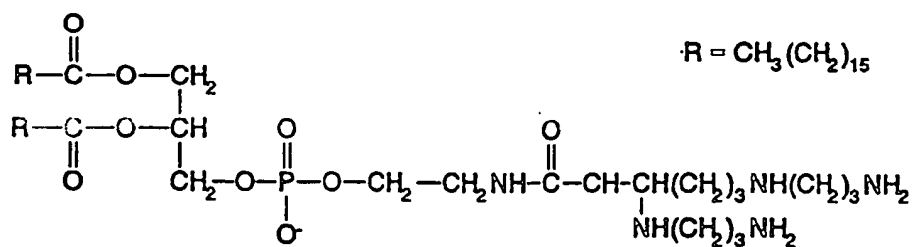
DOGS is available commercially as TRANSFECTAM™ (Promega, Madison, WI).

Another preferred cationic lipid is a cholesterol derivative (DC-Chol) which has been synthesized and formulated into liposomes in combination with DOPE. The structure of this compound is:

$$CH_3-, +$$
$$\quad NH-(CH_2)_2NH-C(=O)-[Cholesterol]$$
$$CH_3-$$
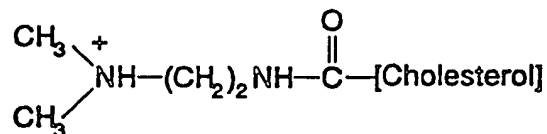
Another preferred cationic lipid is lipopolylysine, which is formed by conjugating polylysine to DOPE.

Additional preferred cationic lipids are described by the general formula:

$$R_1-O-CH_2$$
$$R_2-O-CH$$
$$CH_2-N(Z_1)(Z_2)-(CH_2)_q-X_{1-16}$$

- 18 -

wherein R_1 and R_2 separately or together are C_{1-23} alkyl or

or $-\overset{\overset{\displaystyle O}{\|}}{C}-C_{1-23}$ alkyl or alkenyl, q is 1 to 6,

Z_1 and Z_2 separately or together are H or unbranched alkyl C_{1-6}

X_1 is $-(CH_2)_nBr$, Cl, F or I   n=0-6 or

X_2 is $-(CH_2)_nNH_2$ n=0-6 or

X_3 is $-NH-(CH_2)_m-NH_2$ m=2-6 or

X_4 is $-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$ or

X_5 is $-NH-(CH_2)_3-NH-(CH_2)_4-NH(CH_2)_3-NH2$

X_6 is $-NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH-(CH_2)_3NH_2}{|}}{CH}-(CH_2)_3NH(CH_2)_3-NH_2$

X_7 is $-NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH_2}{|}}{CH}-(CH_2)_3NH_2$

X_8 is $-NH-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle NH-Y}{|}}{CH}-(CH_2)_pNH_2$

where p is 2-5, Y is H or other groups attached by amide or alkyl amino group or

X_9 is a polyamine, e.g., polylysine, polyarginine, polybrene, histone or protamine or

X_{10} is a reporter molecule, e.g., $-NH-\overset{\overset{\displaystyle O}{\|}}{C}-$fluorescein, biotin, folic acid or PPD, or

X_{11} is a polysaccharide or substituted polysaccharide, or

X_{12} is a protein or

X_{13} is an antibody or

X_{14} is an amine or halide reactive group or

- 19 -

X_{15} is $-(CH_2)_r$-SH where r is 0-6 or

X_{16} is $-(CH_2)_s$-S-S-$(CH_2)_t$-NH_2 where s is 0-6 and t is 2-6.

These compounds are useful either alone, or in combination with other
5   lipid aggregate-forming components (such as DOPE or cholesterol) for
formulation into liposomes or other lipid aggregates. Such aggregates are
cationic and able to complex with anionic macromolecules such as DNA or
RNA.

10   *II.*   *Expression Vectors*

One form of polynucleotide which can be used for genetic
immunization is a plasmid expression vector for the expression of the
immunogen protein in eukaryotic cells. The eukaryotic expression vector
15   comprises four main components. First, the plasmid must contain prokaryotic
sequences which code for a bacterial replication origin and an antibiotic
resistance marker. These prokaryotic sequences allow the propagation and
selection of the plasmid within the bacterial host. Second, the plasmid must
contain eukaryotic elements which control initiation of transcription. These
20   elements include promoter and, possibly, enhancer sequences. Third, the
plasmid must contain sequences involved in the processing of transcripts, such
as polyadenylation sequences. Fourth, the plasmid must contain DNA
sequences coding for the immunogen. These DNA sequences may be either
genomic DNA sequences, or complementary DNA (cDNA) sequences. (For
25   reviews of expression vectors, *see* Old *et al., supra*; Sambrook *et al.,*
Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor
Laboratory Press, 1989; Gorman, "High Efficiency Gene Transfer into
Mammalian Cells," in *DNA Cloning, Volume II*, Glover, D.M., Ed., IRL
Press, Washington, D.C., pp. 143-190 (1985).)
30   DNA or cDNA molecules which encode an immunogen can be
operably linked into the expression vector. Two DNA sequences (such as a

promoter region sequence and an immunogen encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the immunogen encoding gene sequence, or (3) interfere with the ability of the immunogen gene sequence to be transcribed by the promoter region sequence.

A DNA sequence encoding an immunogen molecule may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or stagger-ended termini for ligation, restriction digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. In addition, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) may be employed, wherein the prokaryotic promoter is regulated by a eukaryotic promoter (for example, *see* Zhou *et al.*, *Mol. Cell. Biol.* *10*:4529-4537 (1990); Kaufman *et al.*, *Nucl. Acids Res.* *19*:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

The expression of the desired immunogen molecule in animals requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include the promoter of the mouse

- 21 -

metallothionein I gene (Hamer *et al., J. Mol. Appl. Gen. 1*:273-288 (1982));
the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the
SV40 early promoter (Benoist *et al., Nature (London)* 290:304-310 (1981));
the Rous sarcoma virus promoter (Gorman *et al., supra*); and the
cytomegalovirus promoter (Foecking *et al., Gene 45*:101 (1980)).

As is widely known, translation of eukaryotic mRNA is initiated at the
codon which encodes the first methionine. For this reason, it is preferable to
ensure that the linkage between a eukaryotic promoter and a DNA sequence
which encodes the desired immunogen molecule does not contain any
intervening codons which are capable of encoding a methionine (i.e., AUG).
The presence of such codons results either in the formation of a fusion protein
(if the AUG codon is in the same reading frame as the desired receptor
molecule encoding DNA sequence) or a frame-shift mutation (if the AUG
codon is not in the same reading frame as the desired receptor molecule
encoding sequence).

The desired immunogen molecule encoding sequence and an operably
linked promoter may be introduced into the cells of the vaccinee either as a
non-replicating DNA (or RNA) molecule, which may either be a linear
molecule or, more preferably, a closed covalent circular molecule. Since such
molecules are incapable of autonomous replication, the expression of the
desired receptor molecule may occur through the transient expression of the
introduced sequence. Alternatively, permanent expression may occur through
the integration of the introduced sequence into the host chromosome.

Preferably, the introduced sequence will be incorporated into a plasmid
or viral vector capable of autonomous replication in the recipient host.
Several possible vector systems are available for this purpose. One class of
vectors utilize DNA elements which provide autonomously replicating extra-
chromosomal plasmids, derived from animal viruses such as bovine papilloma
virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors
relies upon the integration of the desired gene sequences into the host
chromosome. Additional elements may also be needed for optimal synthesis

of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, *Mol. Cell. Biol.* *3*:280 (1983), and others.

5   Alternatively, the polynucleotide molecule can be an RNA molecule which codes for the desired immunogen. Sufficient quantities of such RNA molecules may be obtained using *in vitro* transcription, followed by RNA purification. The technique of transcribing cloned DNA sequences *in vitro* using DNA-dependent RNA polymerases is well-known in the art (for

10   example, *see Sambrook et al., supra*).

Any immunogen-encoding sequence can be used in this invention. For example, such immunogens include herpes simplex virus glycoprotein D, hepatitis B surface antigen, influenza virus haemagglutinin, and human immunodeficiency virus envelope antigen. In addition, the claimed invention

15   may be used to characterize the protein product of a polynucleotide sequence of unknown identity, as described below.

III.   *Use of the Lipid/Polynucleotide Complex*

20   According to the present invention, the lipid/polynucleotide complex is used to carry out an *in vivo* transfection. Transfected cells express the protein encoded by the polynucleotide, and may present the foreign protein on the cell surface. As a result, the host animal mounts an immune response to the foreign protein, or immunogen.

25   Thus, the lipid/polynucleotide complex can be used as a vaccine to induce active immunity. Preferably, such active immunity is induced in humans, although the invention is not intended to be so limiting. Any animal which may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed

30   invention.

- 23 -

Genetic immunization may be performed by administering vaccines comprising the cationic lipid and polynucleotide in a wide range of dosages, and over a wide range of ratios. Effective dosages and formulations will depend upon a variety of factors (such as the species of the vaccinee), and can

5   be determined by one of ordinary skill in the art. Illustrative dosages, formulations, and modes of administration are provided below.

Cationic lipid-polynucleotide complexes are formed by mixing a cationic lipid solution with an equal volume of polynucleotide solution. The cationic lipid and polynucleotides can be dissolved in any sterile

10   physiologically-compatible aqueous carrier. Preferably, cationic lipid and polynucleotides are dissolved in sterile saline (150 mM NaCl). The solutions are mixed at ambient temperatures. Preferably, the solutions are mixed at 25 °C. After mixing, the cationic lipid-polynucleotide complexes are incubated at room temperature, preferably for 15 to 45 minutes.

15   Administration of lipid/polynucleotide complexes of the present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, or any other suitable means. The specific dosage administered may be dependent upon the age, weight, kind of current treatment, if any, and nature of the immunogen which will be expressed. The initial dose may be

20   followed by a booster dosage after a period of about four weeks to enhance the immunogenic response.

Since genetic immunization generates the production of immunogen-specific antibodies in the vaccinee, the present invention is also directed to methods of producing immunogen-specific antibodies. Polyclonal antibodies

25   may be isolated and purified from vaccinated animals using procedures well-known in the art (for example, see Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988).

This invention is also directed to the use of genetic immunization to produce monoclonal antibodies. According to this method, mice are injected

30   with a lipid/polynucleotide complex, and B-lymphocytes are isolated from the immunized mice. Monoclonal antibodies are produced following the

- 24 -

procedure of Köhler and Milstein (*Nature 256*:495-497 (1975) (for example, *see* Harlow *et al., supra*). Briefly, monoclonal antibodies can be produced by immunizing mice with a cationic lipid-polynucleotide complex, verifying the presence of antibody production by removing a serum sample, removing

5   the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce anti-immunogen antibody, culturing the anti-immunogen antibody-producing clones, and isolating anti-immunogen antibodies from the hybridoma cultures.

10   As an alternative to generating monoclonal antibodies to known immunogens, genetic immunization can be used to identify antigenic determinants in a protein by epitope mapping. According to this method, the polynucleotide of the lipid/polynucleotide complex codes for a portion of a protein molecule. Preferably, random fragments of the DNA encoding the

15   complete protein molecule are generated using sonication (Deininger *et al., Anal. Biochem. 129*:216-223 (1983)) or partial DNase I digestion (Anderson *et al., Nucleic Acids Res. 9*:3015-3027 (1981)), and cloned by blunt-end ligation into a suitable site of an expression vector. Alternatively, DNA fragments for epitope mapping can be obtained by treating DNA molecules

20   with one or more restriction endonucleases, or by using the polymerase chain reaction to synthesize DNA molecules. The generation of monoclonal antibodies by cells derived from the immunized mice will indicate which segments of the protein molecule are immunogenic.

In addition, the claimed invention may be used to characterize the

25   protein product encoded by a DNA or RNA sequence of unknown identity. For example, a genomic library can be constructed in a cosmid vector, wherein the expression of the cloned DNA fragments is regulated by a promoter. The genetic immunization technique can then be used to immunologically characterize the protein products of the subcloned genomic

30   fragments.

- 25 -

As described above, genetic immunization protocols in which naked DNA is administered can require as much as 100 μg of a DNA construct per inoculation. In contrast, the use of cationic lipids as a carrier for DNA constructs according to the claimed invention permits genetic immunization with as little as 5 μg of a DNA construct. Thus, the claimed invention provides a more efficient means of genetic immunization.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

Evaluation of the Immunization Protocol

This series of experiments employed pSV2CAT plasmids which carry the bacterial chloramphenicol acetyl transferase (CAT) gene under the control of the simian virus 40 promoter (SV; Gorman, "High Efficiency Gene Transfer into Mammalian Cells," in *DNA Cloning, Volume II*, Glover, D.M., Ed., IRL Press, Washington, D.C., pp. 143-190 (1985)). Plasmid DNA was isolated from bacterial cells by the alkaline lysis method, and purified by isopycnic centrifugation in cesium chloride/ethidium bromide gradients (Maniatis *et al.*, *supra*). These experiments employed LIPOFECTAMINE™ (BRL) as the cationic lipid. LIPOFECTAMINE™ is composed of 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), which is formulated with dioleoylphosphatidyl ethanolamine (DOPE) at a 3:1 (W:W) ratio in water. The cationic lipids and plasmids were dissolved in sterile saline (150 mM NaCl).

Lipid/DNA complexes were administered to mice by intraperitoneally (IP) or intranasally (IN). For IN administration, lipid/DNA mixtures were formed by mixing 25 μl of a DNA solution with 25 μl of a lipid solution. For IP administration, lipid/DNA mixtures were formed by mixing 200 μl of a

DNA solution with 200 μl of a lipid solution. In these experiments, 5 μg of pSV2CAT were mixed with 0, 15, 30, 45, or 60 μg of lipid. After mixing, the lipid/DNA complexes were allowed to sit at room temperature for 15-45 minutes before administration. As a control, certain mice were injected subcutaneously (SC) with CAT protein (25 μg) in complete Freund's adjuvant.

A CAT enzyme-linked immunoassay (ELISA) was used to determine the presence of CAT antibodies in mouse sera samples. Microwell titer plates were prepared by incubating each well of the plate with 1 μg/ml of CAT in 0.1 M sodium carbonate buffer, pH 9.5. The plates were incubated for 18 hours at 4°C. The wells were blocked with 0.2% ovalbumin dissolved in phosphate-buffered saline (PBS) with 0.1% Tween-20 (dilution buffer). Samples were diluted in the dilution buffer, and 200 μl were added to a well on the plate. The plate was sealed, and then incubated for 60 minutes at 37°C. Following a wash, the plates were incubated with 0.1 μg/ml goat anti-mouse IgG - horseradish peroxidase (HRP) conjugate. The plate was sealed and then incubated for 30 minutes at 37°C. Following a second wash, the plates were developed with 3,3',5,5'-tetramethylbenzidine substrate at room temperature. The reaction was stopped by adding 2N sulfuric acid.

Mice which were immunized with lipid/CAT protein developed an immune response, although it was not as great as the response generated by administration of CAT protein in complete Freund's adjuvant. Several mice injected IP with lipid/pSV2CAT generated an immune response which was weak, but clearly above background. Immunization by intranasal administration gave results that varied among mice, but with a response that was greater than that seen with IP administration.

- 27 -

EXAMPLE 2

Comparison of Cationic Lipids as DNA Complexing Agents

5   These experiments compared the following cationic lipids: LIPOFECTAMINE™, LIPOFECTACE™ (BRL), DORI-ether (VICAL, Inc., San Diego, CA), DORI-ether/lysolipid (VICAL Inc., San Diego, CA), and a bromo lipid (1-propanaminium, N-[2(2-bromo)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-bromide). LIPOFECTACE™ is a 1:2.5 (W/W) liposome

10   formulation of dimethyldioctadecylammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE). The bromo lipid was prepared as described in co-pending U.S. application serial no. 07/937,508 (filed August 28, 1992), which is fully incorporated by reference.

          Immunizations were performed by using IP administration of 5, 10, 20,

15   40, or 80 μg of pSV2CAT, which was complexed with a cationic lipid in a 1:4 ratio (W/W; pSV2CAT/cationic lipid). One mouse received pSV2CAT only, one mouse received CAT protein only, and one mouse received CAT protein in complete Freund's adjuvant. At about day 14, selected mice were given a booster shot.

20   The results were analyzed using Western Blot analysis. Nitrocellulose sheets were prepared by applying purified CAT protein to the nitrocellulose (0.1 μg CAT protein/cm nitrocellulose). Sera samples were diluted into dilution buffer and 2 ml were added to each nitrocellulose strip. The strips were incubated for 90 minutes with rocking at room temperature. Following

25   a wash, the strips were incubated with 0.1 μg/ml goat anti-mouse IgG-alkaline phosphatase conjugate. The strips were then incubated for 30 minutes at room temperature. The strips were developed with 1 ml of nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate stable mix substrate. The strips were rinsed with distilled water following development and dried.

30   The results demonstrated that mice immunized with DNA and lipid produced anti-CAT antibodies. The booster shot did not seem to greatly

- 28 -

enhance the response. Western blot analysis showed that although the ELISA results indicated different intensities of immune response, all the animals injected with lipid-DNA complexes generated anti-CAT IgG.

5

EXAMPLE 3

Effect of Different Promoters on the Immune Response

10   These experiments used the bromo lipid (described in Example 2) or LIPOFECTACE™ as the cationic lipid. The CAT plasmids comprised either the cytomegalovirus (CMV) promoter (Foecking *et al.*, *Gene 45*:101 (1980)), the Rous sarcoma virus (RSV) promoter (Gorman, "High Efficiency Gene Transfer into Mammalian Cells," in *DNA Cloning, Volume II*, Glover, D.M.,

15   Ed., IRL Press, Washington, D.C., pp. 143-190 (1985)), or the SV40 promoter (described above). Mice were immunized IP, as described above, following the protocol:

Lipid	Lipid quantity	DNA quantity
Bromo Lipid or LIPOFECTACE™	20 μg	5 μg
	40 μg	10 μg
	80 μg	20 μg

20

One set (i.e., two mice) were used to test each promoter. As controls, mice received DNA without lipid, CAT protein in complete Freund's adjuvant, or

25   no inoculation.

          The results of ELISA analysis indicated that the SV40 promoter seemed to work the best followed by the CMV promoter and then, the RSV promoter. The bromo lipid gave more consistent results, compared with LIPOFECTACE™, in generating an immune response. The positive results

30   observed in the ELISA was confirmed by Western Blot analysis. In both assay formats, the results obtained with DNA-lipid immunizations were greater than the results observed with DNA alone.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following Claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in their entirety.

- 30 -

What Is Claimed Is:

1.   A method for eliciting an immune response in an animal, comprising the steps of:

      (a)   mixing at least one cationic lipid with a polynucleotide, coding for an antigenic determinant, thereby forming a cationic lipid-polynucleotide complex; and

      (b)   administering said lipid-polynucleotide complex to said animal.

2.   The method of claim 1, wherein said cationic lipid is LIPOFECTAMINE™.

3.   The method of claim 1, wherein said cationic lipid is LIPOFECTACE™.

4.   The method of claim 1, wherein said cationic lipid is 1-propanaminium, N-[2(2-bromo)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-bromide.

5.   The method of claim 1, wherein said cationic lipid is DORI-ether.

6.   The method of claim 1, wherein said cationic lipid is DORI-ether lysolipid.

7.   The method of claim 1, wherein said polynucleotide is an RNA molecule, wherein said RNA molecule codes for an immunogen.

8. The method of claim 1, wherein said polynucleotide is a recombinant DNA molecule comprising:

    (a)   a promoter; and

    (b)   a DNA sequence coding for an immunogen, wherein the transcription of said DNA sequence is under the control of said promoter.

9. The method of claim 1, wherein said polynucleotide is a recombinant RNA molecule comprising:

    (a)   a promoter; and

    (b)   an RNA sequence coding for an immunogen, wherein the expression of said RNA sequence is under the control of said promoter.

10. The method of claim 8, wherein said recombinant DNA molecule is an expression vector.

11. The method of claim 10, wherein said promoter is an SV40 promoter.

12. The method of claim 10, wherein said promoter is an RSV promoter.

13. The method of claim 10, wherein said promoter is a CMV promoter.

14. A method for producing polyclonal antibodies to an immunogen in an animal, comprising the steps of:

    (a)   mixing at least one cationic lipid with a polynucleotide, coding for said immunogen,

- 32 -

          thereby forming a cationic lipid-polynucleotide complex;

    (b)   administering said lipid-polynucleotide complex to said animal; and

5   (c)   isolating said polyclonal antibodies from said animal.

    15.   A method for producing monoclonal antibodies comprising the steps of:

10   (a)   mixing at least one cationic lipid with a polynucleotide, thereby forming a lipid-polynucleotide complex, wherein said polynucleotide comprises a DNA sequence coding for an immunogen;

    (b)   administering said lipid-polynucleotide complex to at least one mouse;

15

    (c)   removing B-lymphocytes from said immunized mice;

    (d)   fusing said B-lymphocytes from said immunized mice with myeloma cells, thereby producing hybridomas;

    (e)   cloning said hybridomas;

20   (f)   selecting positive clones which produce anti-immunogen antibody;

    (g)   culturing said anti-immunogen antibody-producing clones; and

    (h)   isolating anti-immunogen antibodies from said cultures.

25

    16.   A method for mapping the epitopes of a protein molecule, comprising the steps of:

    (a)   fragmenting DNA molecules coding for said protein molecule in a random manner;

30   (b)   subcloning said DNA fragments in an expression vector;

(c)   mixing at least one cationic lipid with each of said expression vector subclone, thereby forming a cationic lipid-expression vector complex with each of said expression vector subclone;

(d)   administering said cationic lipid-expression vector complexes to mice; and

(e)   determining which of said DNA fragments are capable of generating the production of antibodies in said mice.

17.   A method for generating active immunity against an infectious disease in an animal, comprising the steps of:

(a)   mixing at least one cationic lipid with a polynucleotide, coding for an antigenic determinant of an organism which is the causative agent of said infectious disease, thereby forming a cationic lipid-polynucleotide complex; and

(b)   administering said lipid-polynucleotide complex to said animal; whereby active immunity to said infectious disease is generated.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :424/9, 420; 435/69.1, 69.6, 70.21, 172.1, 172.2, 172.3; 514/44; 530/387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9, 420; 435/69.1, 69.6, 70.21, 172.1, 172.2, 172.3; 514/44; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, APS, EMBASE, CHEMICAL ABSTRACTS
search terms: genetic immunization, cationic lipid, liposome, epitope mapping, polyclonal antibody, monoclonal antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A. JOHNSTONE et al, "IMMUNOCHEMISTRY IN PRACTICE" published 1987 by Blackwell Scientific Publications (Oxford), pages 30-47, see the entire document.	14, 15
Y	Journal of General Virology, Volume 71, issued 1990, A.R. Neurath et al, "B cell epitope mapping of human immunodeficiency virus envelope glycoproteins with long (19- to 36-residue) synthetic peptides", pages 85-95, see the entire document.	16
Y	Nature, Volume 349, issued 24 January 1991, P.L. Felgner et al, "Gene therapeutics", pages 351-352, see the entire document.	1-17

| ☒ | Further documents are listed in the continuation of Box C. | ☐ | See patent family annex. |

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 AUGUST 1994	30 AUG 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	BRUCE CAMPELL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, USA, Volume 90, issued May 1993, B. Wang et al, "Gene inoculation generates immune responses against human immunodeficiency virus type 1", pages 4156-4160, see the entire document.	1-17
Y	Science, Volume 259, issued 19 March 1993, J.B. Ulmer et al, "Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein", pages 1745-1749, see the entire document.	1-17
Y	Nature, Volume 356, issued 12 March 1992, D. Tang et al, "Genetic immunization is a simple method for eliciting an immune response", pages 152-154, see the entire document.	1-17
Y	Nature, Volume 337, issued 26 January 1989, P.L. Felgner et al, "Cationic liposome-mediated transfection", pages 387-388, see the entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06105

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
                          ☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 25/26, 25/28, 43/04; A61K 31/70, 35/14, 49/00; C07K 3/00, 13/00, 15/00; C12N 15/00; C12P 21/02, 21/06; G01N 31/00, 33/48

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-13 and 17, drawn to methods for eliciting an immune response and generating active immunity against an infectious disease in an animal.
Group II, claim(s) 14-15, drawn to methods for production of polyclonal and monoclonal antibodies.
Group III, claim(s) 16, drawn to a method for epitope mapping.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The three inventions are drawn to divergent methods.
Invention I is distinct from invention II because II requires materials and processes not required for the method of I, such as isolation of antibodies and production of hybridomas.
Invention III is distinct from each of I and II because the method of III requires procedures not required for I and II, such as subcloning of DNA fragments and injection of multiple DNA fragments.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.